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Short sequence-paper

A possible involvement of melanocortin 3 receptor in the regulation of adrenal gland function in the chicken

Sakae Takeuchi *, Sumio Takahashi

Department of Biology, Faculty of Science, Okayama University, 3-1-1, Tsushimanaka, Okayama 700-8530, Japan

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Abstract

The melanocortin 3 receptor (MC3-R) in the melanocortin receptor family has been identified as a neural receptor subtype mainly expressed in the brain in mammals. We report here the isolation of the chicken gene for MC3-R, CMC3, displaying different tissue distribution from mammalian counterparts. The CMC3 gene was found to be a single copy gene encoding a 325 amino acid protein, sharing 75.3–76.8% identity with mammalian counterparts. When assessed by RT-PCR, the CMC3 mRNA was not detected in the brain but was exclusively expressed in adrenal glands, where Agouti-related protein/Agouti-related transcript (AGRP/ART), a newly identified endogenous antagonist of MC3-R, is expressed in mammals, raising the possibility that the CMC3 plays a role in complicated regulation of the gland function by melanocortins and AGRP/ART in the chicken. Noteworthy, MC1-R gene was found to be a quite unique member of the chicken MC-R family with regard to GC content and codon usage. It may reflect as yet unidentified evolutionary pressure operating specifically on the gene.

Keywords: Melanocortin 3 receptor; Genomic sequencing; Tissue distribution; Codon usage; GC content; (Chicken)

Melanocortin peptides are generated from a common precursor glycoprotein, the proopiomelanocortin (POMC), by post-translational processing [1]. These peptides include adrenocorticotropic hormone (ACTH) and melanocyte-stimulating hormones (α-, β-, and γ-MSH). The POMC gene is expressed mainly in the anterior and intermediate lobes of the pituitary and in the arcuate nucleus of the hypothalamus, but at a lower level also in a wide variety of peripheral tissues and of brain regions [2]. Mammalian melanocortins are now widely recognized to have a broad array of physiological actions both in the central nervous system and in the periphery, coincident with their wide distributions of expression and those of their corresponding receptors [2,3]. Five subtypes of melanocortin receptors (MC-Rs) have been identified so far with their discrete pharmacological properties and tissue distributions [3]. Two endogenous antagonists of those receptors have been identified, Agouti [4–7] and Agouti-related protein/Agouti-related transcript (AGRP/ART) [8,9], acting in a paracrine manner to regulate MC-Rs function. Thus, great progress has been made in the understanding of the molecular mechanisms of melanocortin actions in mammals. However, little is known about the physiological role of avian melanocortins.

Recently, we have isolated four receptor genes be-

* Corresponding author. Fax: +81 (86) 2517876; E-mail: stakeuch@cc.okayama-u.ac.jp

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longing to the MC-R family in birds, the CMC1 [10,11], CMC2[12], CMC4 and CMC5 [13], which are chicken homologues of mammalian MC1 (MSH)-R, MC2 (ACTH)-R, MC4-R and MC5-R, respectively. Based on their distributions of expression, melanocortins have been suggested to affect a variety of functions both in the brain and peripheral tissues in the chicken as in mammals, although avian pituitary glands do not possess a distinct intermediate lobe, the main source of circulating α-MSH in most vertebrates.

The present study was aimed to identify further possible target organs of melanocortins by cloning of, and localizing the expression of the remaining member of the MC-R family, the MC3-R, which has been identified only in mammals [14–16].

DNA fragments encoding a part of the mouse MC3-R were obtained by PCR using genomic DNA of an ICR mouse as a template. The primers designed from the previous report on the mouse MC3-R sequence [16] were AACCTGCACTCTCCCATGTACTTCT and GATGACGGAGTTGCACATGATGAG. The PCR-derived clone, designated as pcrmMC3-R, was 681 bp in length and contained a part of the coding sequence identical to that of the reported mouse MC3-R [16]. Using pcrmMC3-R as a probe, we performed a Southern blotting to detect MC3-R-related genes in the genome of the domestic chicken, the Rock Cornish. As shown in Fig. 1, the probe hybridized under low stringent conditions with at least two species of DNA fragments digested with *Eco*RI, *Bam*HI, or *Pst*I. The restriction pattern indicated by open arrowheads was identical to that of the CMC1 gene ([10,11] and personal communications), and that indicated by solid arrowheads differed from those of any of the chicken MC-R genes we have cloned previously [10–13]. Therefore, it is highly probable that the former corresponds to the CMC1 gene, and the latter represents the chicken MC3-R gene. Detecting the chicken MC3-R gene as a single band in each digestion suggests that the gene is a single copy gene. Considering our previous studies on the chicken MC-R family, it is likely that the chicken melanocortin activities are mediated by five subtypes of MC-Rs as mammals.

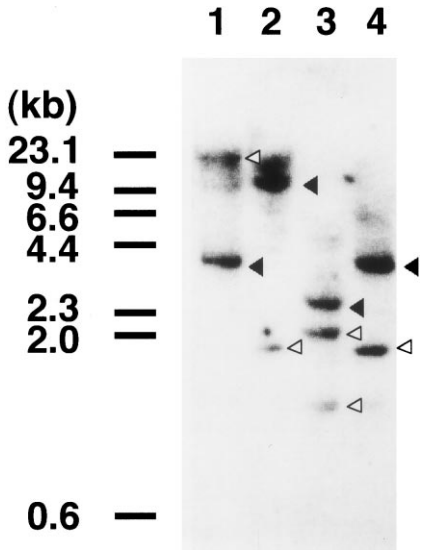

Fig. 1. Detection of MC3-R-related receptor genes in the chick genome by Southern blotting. Genomic DNA (10 μg) prepared from 10 day chick embryos (Rock Cornish) was digested with *Eco*RI (lane 1), *Bam*HI (lane 2), *Pst*I (lane 3), or *Eco*RI and *Bam*HI (lane 4) and the resulting products were subjected to a Southern blot analysis using pcrmMC3-R as a probe. Radiolabeled probe was prepared using a Random Primer DNA labeling Kit ver. 2 (Takara) and [α-^{32}P]dCTP (Amersham). After the final low stringent wash (0.1% SDS in 2×SSC, 30 min at 60°C), the blot was exposed to a RX-U X-ray film (Fuji film, Japan) for 48 h at −80°C with double intensifying screens. The sizes of the DNA fragments were determined by comparison with *Hin*dIII-digested λ DNA fragments. The locations of the λ DNA fragments are shown at left. Open and solid arrowheads indicate CMC1 and the chicken MC3-R genes, respectively.

A genomic DNA library derived from the liver of the White Leghorn chicken was screened using pcrmMC3-R as a probe. One of the positive clones contained the corresponding *Eco*RI fragment (approx. 3.5 kb in length) as observed in the genomic Southern blotting. The DNA fragment was subcloned and partial sequence was determined. The cloned DNA fragment was found to contain an open reading frame of 978 bp, beginning with ACCATGA which is in agreement with the Kozak consensus sequence for translation initiation sites [17] (Fig. 2). A typical TATA box was recognized in the 5′-untranslated flanking region. This sequence is available from DDBJ, EMBL, and GenBank data libraries under accession No. AB017137.

The predicted protein, designated as CMC3, consisted of 325 amino acid residues with seven hydrophobic domains, a characteristic of G-protein-coupled receptors [18]. The N-terminal extracellular domain contained three potential sites for N-linked glycosylation. Six potential sites for phosphorylation

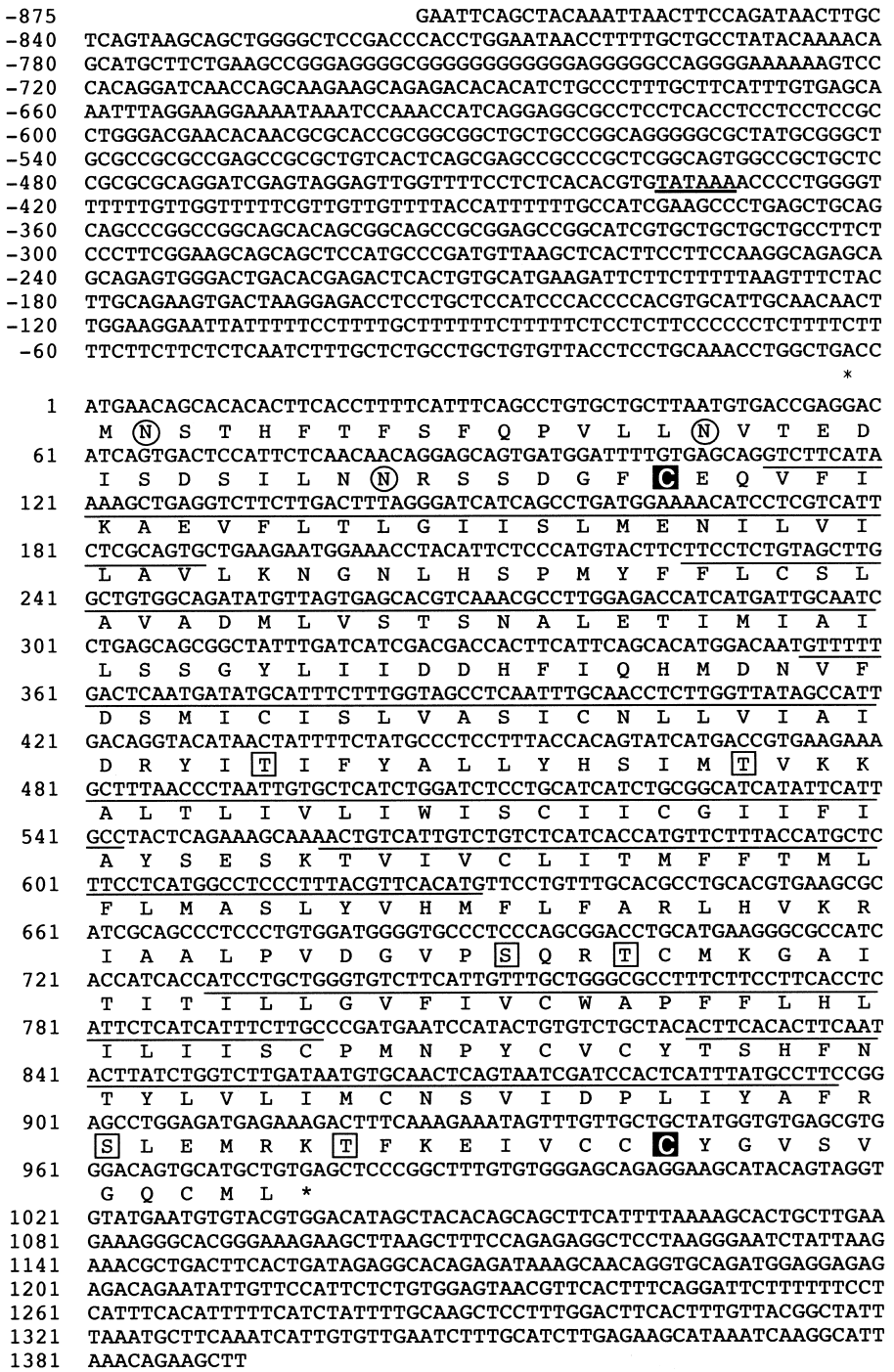

Fig. 2. Nucleotide and predicted amino acid sequences of the chick MC3-R, CMC3. Transmembrane segments indicated by overlines are determined by hydropathy analysis [22]. In the 5′ non-coding region, a typical TATA box is indicated by underline. Possible sites for *N*-glycosylation and phosphorylation are indicated by circles and squares, respectively. White letters indicate the cysteine residues conserved in all cloned MC-Rs.

by the cAMP-dependent protein kinase and/or protein kinase C [19] were also recognized in intracellular domains. There were two cysteine residues in the N-terminal extracellular and C-terminal intracellular domains, both of which are conserved in all cloned MC-Rs [13] and are considered to form a disulfide bond with one of the cysteine residues in the third extracellular loop and to be the site for palmitoyla-

tion, respectively. A homology search on an EMBL protein database revealed the highest similarity of CMC3 to mammalian MC3-Rs; CMC3 shared 76.8%/315 aa, 75.3%/312 aa, and 75.3%/312 aa identity with the human [14], mouse [16] and rat [15] MC3-Rs, respectively. Judging from these results, CMC3 was concluded to be the chicken homologue of mammalian MC3-R.

The alignment of CMC3 with mammalian counterparts is shown in Fig. 3. CMC3 showed a high degree of identity with the human, mouse and rat MC3-Rs, and most of the potential sites for phosphorylation by the cAMP-dependent protein kinase

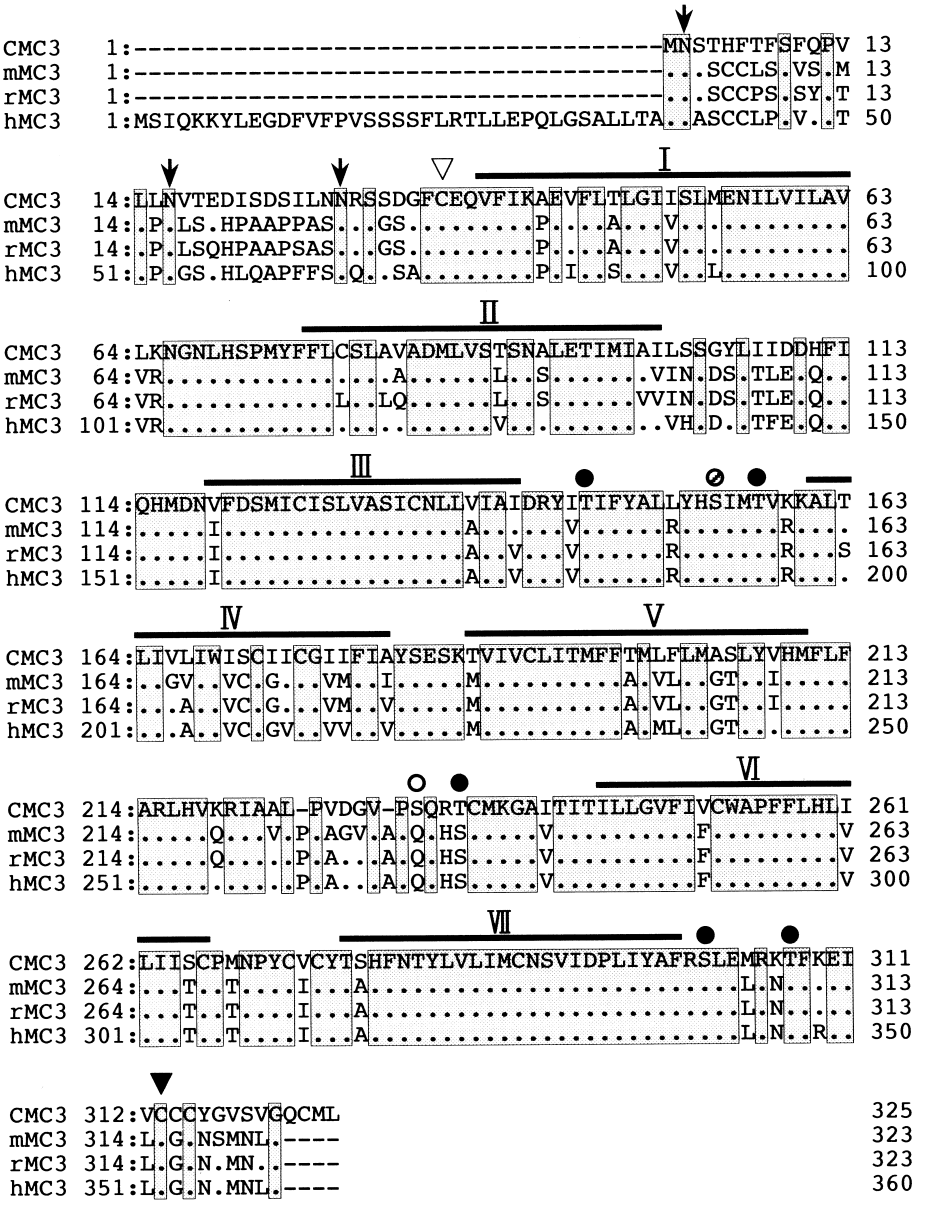

Fig. 3. Alignment of CMC3 with mammalian MC3-Rs. CMC3 sequence is compared to the mouse (mMC3) [16], rat (rMC3) [15] and human (hMC3) [14] MC3-Rs. Amino acid residues identical to CMC3 are indicated by dots and those conserved among all MC3-Rs are marked by shading. The putative transmembrane segments are denoted by overlines and Roman numerals. Possible sites for *N*-glycosylation are indicated by arrows. Phosphorylation sites conserved among all MC3-Rs, specific for CMC3, and conserved among mammalian MC3-Rs are indicated by solid, open and hatched circles, respectively. Open and a solid arrowheads indicate the cysteine residues conserved in all cloned MC-Rs (see text for details).

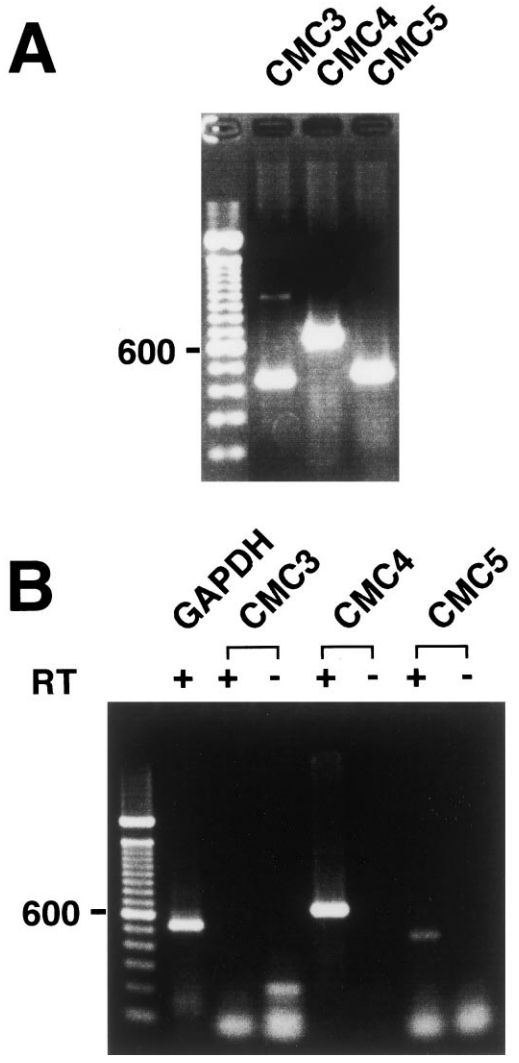

Fig. 4. RT-PCR analysis of CMC3 expression in the brain of adult chicken. (A) Evaluation of PCR primers. Primers specific for each MC-R were designed and tested against genomic DNA (0.8 μg) prepared from 10 day chick embryos (Rock Cornish). The PCR was performed using AmpliTaq Gold DNA polymerase (Perkin Elmer). Each primer pair amplified DNA fragments of the expected length (435, 649 and 448 bp for CMC3, CMC4 and CMC5, respectively) with the same quantity. (B) Detection of MC-Rs expression in the chicken brain. Two μg of brain total RNA from adult White Leghorn chicken was subjected to RT reaction using Superscript II reverse transcriptase (Gibco BRL) according to the manufacturer's directions. One tenth of the reaction was subjected to PCR. As a negative control, 1 μg of total RNA was also subjected to PCR (denoted as RT minus). In each case, a 100 bp ladder was used as a molecular marker. The primers for CMC3 were CGACGACCACTTCATTCAGCACAT and GCAAACAATGAAGACACCCAGCAG, and those for CMC4, CMC5 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were described elsewhere [13].

and/or protein kinase C were conserved. Like in other subtypes of the chicken MC-R family, the N-terminal extracellular region of CMC3 displayed low homology with mammalian counterparts, although all three potential sites for N-linked glycosylation were conserved. It has been demonstrated that the N-terminal extracellular region of human MC-Rs could be deleted without affecting ligand binding or expression levels [20], suggesting that the N-terminal extracellular region of MC-Rs is not structurally and functionally important. Therefore, it is likely that CMC3 has the same or a similar function as mammalian MC3-Rs.

Mammalian MC3-R has been identified as a neural MC-R subtype predominantly expressed in the brain [14–16]. To examine whether or not CMC3 is expressed in the chicken brain, we performed RT-PCR on brain RNA isolated from adult White Leghorn chicken using CMC3 primers of high specificity and efficiency (Fig. 4A). As shown in Fig. 4B, no CMC3 mRNA was detected, while cDNAs for CMC4 and CMC5 as well as GAPDH were obtained by the PCRs, suggesting that the chicken brain does not express CMC3, or does at a level below the sensitivity of our detection system. Alternatively, the expression might be confined to quite limited regions in the chicken. The result also suggests that CMC4 is the predominant subtype in the MC-R family expressed in the chicken brain, although the PCR technique is essentially qualitative.

The tissue distribution of CMC3 expression in adult chicken is shown in Fig. 5. The CMC3 mRNA was detected only in the adrenal gland, suggesting that CMC3 is an adrenal-specific receptor subtype in the chicken. Recently, Agouti-related protein/Agouti-related transcript (AGRP/ART) has been identified as a neuropeptide involved in the hypothalamic control of feeding behavior by antagonism of MC3-R and/or MC4-R in mammals [8,9]. The endogenous antagonist is also produced by the adrenal medulla, which secretes catecholamines such as adrenaline and noradrenaline. Whether AGRP/ART signaling mediated by CMC3 is involved in the regulation of the secretory function of the adrenal gland awaits further analysis; however, it seems possible that CMC3 plays a role in the complicated regulation of the gland function by melanocortins and AGRP/ART in the chicken.

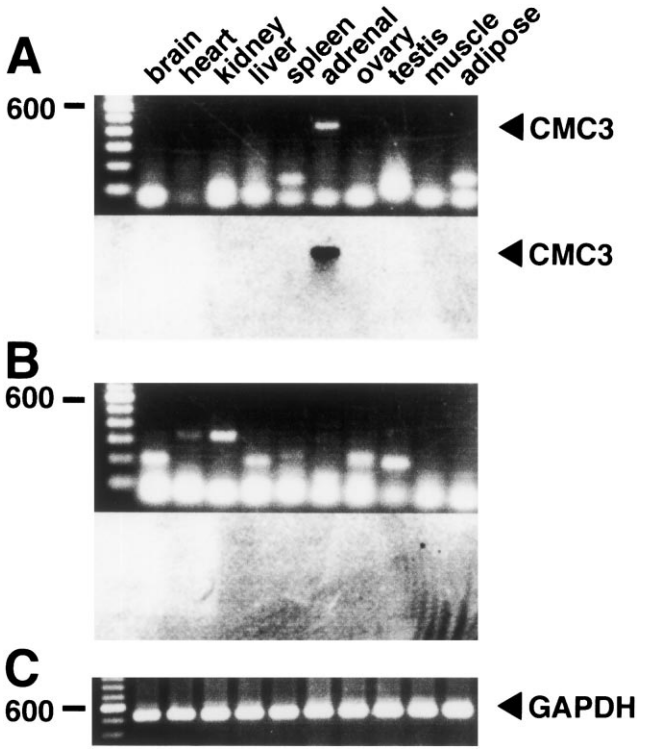

Fig. 5. Tissue distribution of CMC3 expression in adult chicken. Two μg of each total RNA prepared from various tissues of adult White Leghorn chicken was subjected to RT reaction using Superscript II reverse transcriptase (Gibco BRL) according to the manufacturer's directions. One tenth of each reaction was used in PCRs using AmpliTaq Gold DNA polymerase (Perkin Elmer) and specific primers for CMC3 (A) or GAPDH (C). As a negative control, 1 μg aliquot of total RNA was also subjected to PCR using the primers for CMC3 (B). The lower panels in A and B are Southern blots of each gel using pcrmMC3-R as a probe. Labeling and detection of the probe were carried out using an ECL Random Prime Labeling and Detection system (Amersham) according to the manufacturer's directions. In each case, a 100 bp ladder was used as a molecular marker.

The present results and our previous reports [12,13] demonstrated that the chicken adrenal glands express all subtypes of the MC-R family, except for CMC1. Since members of the MC-R family are believed to have evolved by repeated duplication and divergence, the overlapped expression appears not to be accidental, but to be the reflection of evolutionary processes, although mammalian MC3-R is predominantly expressed in the brain. In this sense, it would be of interest to study the expression pattern of MC3-R as well as other subtypes of the MC-R family in lower vertebrates, which might also provide insights for the elucidation of the evolutionary process of melanocortin systems.

The region between the conserved N- and C-terminal cysteine residues showed significant similarities among five members of the chicken MC-R family both in nucleotide and amino acid sequences, ranging from 56.9 to 69.6% and from 42.9 to 73.2%, respectively. To elucidate the molecular phylogenetic relationships among MC-Rs, dendrograms representing sequence similarities among the chicken MC-Rs were constructed by the UPGMA and the neighbor-joining methods. As shown in Fig. 6, the evolutionary trees obtained by those two methods based on amino acid sequences displayed an identical topology with each other, and that based on nucleotide sequences also had a similar topology with a minor difference in CMC3 with respect to CMC4 and CMC5. All these trees indicated that chicken MC-Rs diverged first into CMC2 and other MC-Rs, which subsequently differentiated into CMC1 and a subgroup containing CMC3, CMC4, and CMC5,

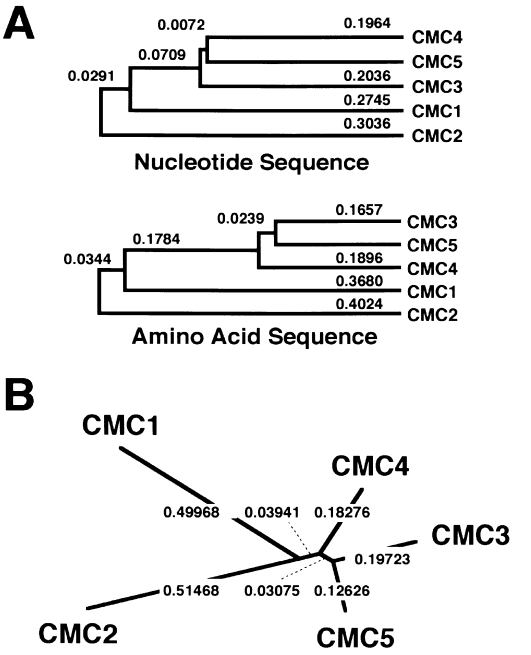

Fig. 6. Molecular phylogenetic trees representing sequence similarities among the MC-R family in the chicken. (A) Dendrograms constructed by the UPGMA method, based on amino acid and nucleotide sequences for the chicken MC-Rs using GENETYX software (Software Development). (B) Phylogenetic tree constructed by the neighbor-joining method, based on amino acid sequence for the chicken MC-Rs using Clustal V software [23].

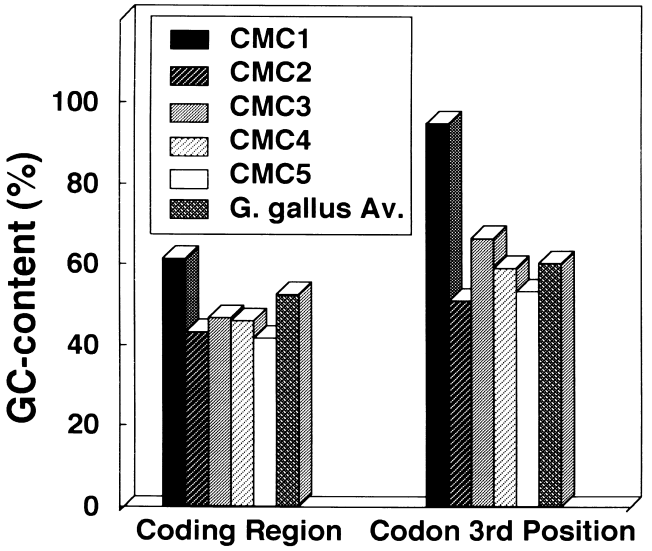

Fig. 7. GC contents in coding region and codon third position in the chicken MC-Rs. G. gallus Av. indicates the average of 1281 chicken genes [21].

suggesting that the newly identified MC-Rs, CMC3, CMC4, and CMC5, are more close to CMC1 than CMC2.

Interestingly, when comparing GC contents in coding sequence and codon third position among those genes, CMC1 was found to be a unique member of the MC-R family with respect to GC content and codon usage (Fig. 7); CMC1 is extremely rich in G/C both in coding sequence and codon third positions [12], where others are equivalent to the average of 1281 chicken genes [21]. It may be the result of as yet unidentified evolutionary pressure operating specifically on CMC1. Localization of the gene on chromosome may provide a clue to elucidate the pressure.

The present study and our previous reports [10–13] demonstrated that the chicken MC-Rs are expressed in a wide variety of tissues, raising the possibility that melanocortins have diverse functions in the chicken. Identification of ligands for those receptors as well as the localization of POMC expression should contribute sufficiently to elucidating and understanding of melanocortin systems in birds.

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